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Prenatal BoBs™ in the cytogenetic analysis of products of spontaneous miscarriage

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Background. Fifty percent of spontaneous miscarriages (SMs) are attributed to chromosomal abnormalities. Cytogenetic analysis is an important tool for patient counselling and assessment of the risk of recurrence in future pregnancies. Conventional karyotyping has been the gold standard for chromosomal investigation of products of conception (POC), but it has limitations due to sample maceration, culture failure and maternal cell contamination. Molecular cytogenetic approaches have therefore been developed and found valuable in the cytogenetic investigation of these samples. The Prenatal BoBs™ and KaryoLite BoBs™, based on the newly developed BACs-on-Beads™ technology, have been described as reliable tests for rapid detection of aneuploidies in prenatal and POC samples, respectively.

Objective. To describe our clinical experience of routine screening of POC samples with Prenatal BoBs™, the test used by our laboratory in France.

Methods. Seventeen samples collected at the University Hospital of Sidi Bel Abbès (Western Algeria) and a further 60 from the University Hospital of Clermont-Ferrand (France) were analysed (19 chorionic villi from products of curettage, 12 placentas, 9 amniotic cells and 37 biopsy specimens). All were screened for the frequent aneuploidies (chromosomes 13, 18, 21, X and Y) in addition to nine microdeletion/microduplication syndrome regions by Prenatal BoBs™. Standard karyotyping was performed on 51 samples, but failed in 38 cases.

Results. Prenatal BoBs™ identified one trisomy 21 and one deletion of 17p13.3. Furthermore, it provided a conclusive result in cases of culture failure (n=38) and in samples with macerated tissue (n=19). The overall failure rate was 11.4%.

Conclusions. Prenatal BoBs™ is a promising technology that represents a fast, sensitive and robust alternative to routine screening for chromosomal abnormality in products of SM. Furthermore, it overcomes the limitations of conventional karyotyping and current molecular cytogenetic techniques.

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Of all diagnosed pregnancies, 10 - 15% end in spontaneous miscarriage (SM).[1] Chromosomal abnormalities are the most common cause, more than 50% of aborted fetuses in the first trimester showing numerical abnormalities including trisomies, X monosomies and tripoidies. Aneuploidies become less prevalent as pregnancy progresses (30% in the second trimester), because most abnormal fetuses have miscarried earlier. A number of other factors (maternal factors, infections, etc.) may also play a role.[2,3]

Cytogenetic analysis of fetal tissue after SM is recommended for prognostic and diagnostic purposes, and to give a better estimate of the risk of recurrence in future pregnancies. It enables detection of possible unbalanced segregation associated with advanced maternal age or the presence of balanced structural rearrangement.[3,4] Conventional karyotyping has been the gold standard for the cytogenetic analysis of products of SM. However, SM products in the first trimester are often collected at advanced stages of maceration as a result of tissue disintegration of the dead fetus *in utero*, which may cause significant DNA damage, or are haemorrhagic samples contaminated with maternal blood. Because karyotype analysis requires cell culture to obtain metaphase spreads, the poor quality of SM products often means that it is impossible to obtain a result because of culture failure, or that results are invalid due to maternal cell contamination (MCC).

Some molecular cytogenetic approaches such as FISH (fluorescence *in situ* hybridisation), MLPA (multiplex ligation probe amplification) and QF-PCR (quantitative fluorescence-polymerase chain reaction) have been described as valuable in the cytogenetic analysis of SM products.[5-7] Performed within 24 - 48 hours, as opposed to weeks for karyotyping, these do not require cell culture, and DNA can be analysed directly after it is extracted. However, while it is possible to investigate several regions with FISH, the number of regions that can be explored with QF-PCR is limited, and MLPA requires the use of two commercial kits to invesigate subtelomere regions.[8] An additional approach is array-comparative genomic hybridisation (CGH), which has a better diagnostic yield than conventional karyotyping in the screening of products of conception (POC)[1] but with the disadvantage of high cost.

A new rapid method for the detection of whole-chromosome aneuploidies, based on a BACs-On-Beads™ technology (Perkin Elmer, Finland), has recently been introduced. Two assays are available, Prenatal BoBs™ for the detection of trisomy 13, 18, and 21 and the most frequent syndromes associated with microdeletions, and KaryoLite BoBs™, which can detect aneuploidy in all chromosomes by quantifying the proximal and terminal regions of each chromosome arm. Prenatal BoBs™ has been shown to be a robust technology for the prenatal investigation of fetuses with or without abnormalities on ultrasound,[5,9] whereas KaryoLite BoBs™ has been found clinically useful for the investigation of SM products.[10]

Since September 2012, Prenatal BoBs™ has been used for prenatal diagnosis in the routine clinical setting in the Medical Cytogenetic Service of the University Hospital of Clermont-Ferrand (France). We describe our clinical experience of the screening of SM products collected at the university hospitals of Clermont-Ferrand and Sidi Bel Abbès (Western Algeria) using Prenatal BoBs™.

Patients and methods

Biological samples and DNA extraction

Seventeen POC were collected after SMs in the maternity department of the Hassani Abdelkader University Hospital of Sidi Bel Abbès, and 60 additional samples were collected at the University Hospital of Clermont-Ferrand after medical abortion due to suspicion of a molar pregnancy (*n*=1), spontaneous abortion (*n*=8) or fetal death *in utero* (*n*=51). Samples included chorionic villi from products of curettage (*n*=19), placenta (*n*=12), amniotic cells (*n*=9) and biopsy specimens (*n*=37).

Gestational ages ranged from 5 to 40 weeks (mean 21.5): 17 specimens were from the first trimester (5 - 12 weeks), 35 from the second trimester (13 - 24 weeks) and 25 from the third trimester (25 - 41 weeks). Women who had SMs were between 19 and 43 years of age (mean 30.3), 23.3% being of advanced maternal age (≥35). All women provided written consent.

Placental chorionic villi from maternal decidua, blood clots and mucus were dissected under a dissecting microscope into fragments a few millimetres in size. DNA was obtained from chorionic villus cells, amniotic cells or fetal skin fibroblasts without cell culture using the QIAamp DNA Mini Kit (Qiagen, Germany).

Prenatal BoBs™ assay

All the samples collected were analysed using the Prenatal BoBs™ kit. Fig. 1 shows the main steps of the assay. Prenatal BoBs™ is a multiplex bead-based suspension array technology designed for gain-and-loss screening of chromosomes 13, 18, 21, X and Y and nine targeted microdeletion regions. It uses the Luminex™ xMAP™ technology (Luminex Corp., USA), a multiplexing technology utilising polystyrene beads approximately 5 µm in diameter that have been impregnated with a specific ratio of two different fluorescent dyes. Ten concentrations of both dyes were used, and a range of >100 different dye/bead combinations with distinct fluorescent signatures or spectral addresses that can be identified through excitation of the impregnated dyes when read by the Luminex™ analyser was created. In Prenatal BoBs™, each bead was coupled with bacterial artificial chromosomes (BACs) derived from chromosome regions of interest. Using a flow cytometry approach, each bead was read by two separate lasers in a Luminex™ 100/200™ analyser equipped with xPONENT 3.1 software. The BoBsoft™ software generated a 'Results tab' with a numerical and graphical representation of the fluorescent probe ratio v. female and male references. A sample was defined as 'normal disomic' when the fluorescent ratio was ~1.0 for all loci analysed.

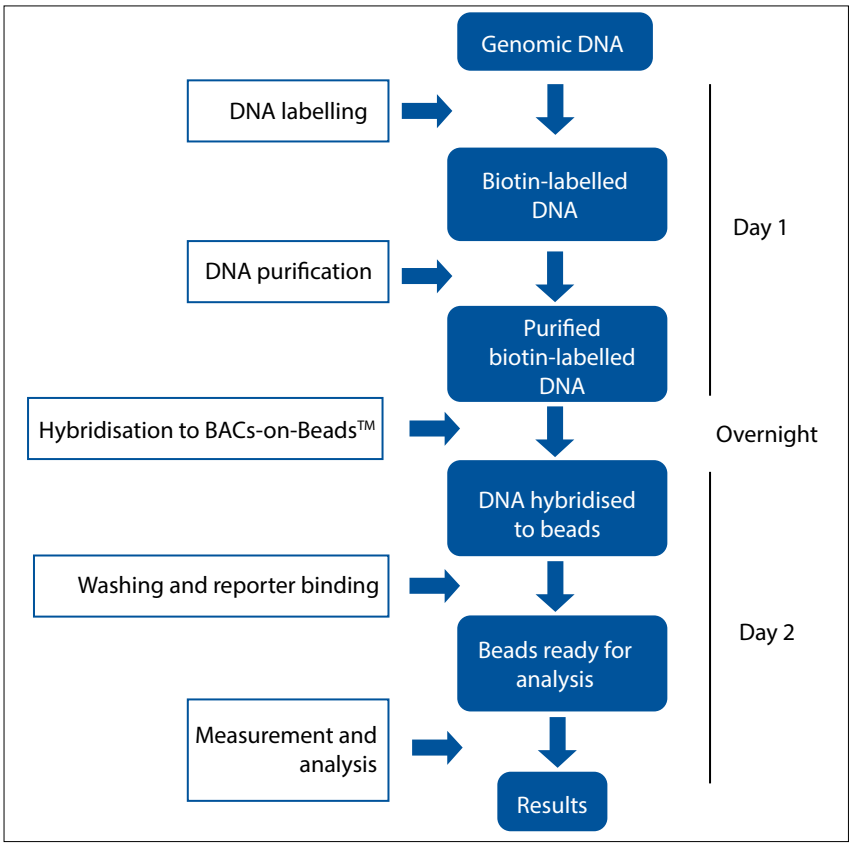

Fig 1. Main steps of the Prenatal BoBs™ assay. Briefly, genomic DNA is labelled with biotin, purified and hybridised to BACs-on-Beads™ probes. Then the reporter molecule (streptavidin-phycoerythrin) is bound to biotin-labelled DNA. Thereafter, fluorescent signals are measured with the Luminex™ 100/200 and data are analysed with BoBSoft™ software.

Table 1. Microdeletion syndromes with the chromosomal regions targeted by Prenatal BoBs™

Aneuploidies/microdeletion syndromes	Target chromosomal region	Number of probes
Wolf-Hirschhorn syndrome	4p16.3	5
Cri du chat syndrome	5p15.3-p15.2	8
Williams-Beuren syndrome	7q11.2	5
Langer-Giedon syndrome	8q23-q24	7
Prader Willi/Angelman syndrome	15q11-q12	7
Miller-Dieker syndrome	17p13.3	6
Smith-Magenis syndrome	17p11.2	4
Di George 1 syndrome	22q11.2	4
Di George 2 syndrome	10p14	4
Patau syndrome (trisomy 13)	13q13.3-q21.2	5
Edwards syndrome (trisomy 18)	18p11.32-q22.1	5
Down syndrome (trisomy 21)	21q22.11-q22.3	5
Aneuploidy of chromosome X	Xp22.31-q27.3	5
Aneuploidy of chromosome Y	Yp11.2-q11.23	5

Where deletions or duplications were present, probe ratios were outside the normal expected ratio range determined by the software for each sample.

The contents of the Prenatal BoBs™ kit were sufficient for 96 reactions. This assay was used for the rapid detection of gains and losses of DNA in 75 regions, including aneuploidies of chromosomes 13, 18, 21, X and Y, as well as gains and losses in nine microdeletion syndrome regions that are often associated with genetic disorders. Eighty-three BAC probes were used in the Prenatal BoBs™ panel: five for each of chromosomes 13, 18, 21,

X and Y, four to eight each for the nine well-defined target loci, and six autosomal controls, as shown in Table 1.

Selection of the nine microdeletion syndrome regions was based on their association with chromosomal disorders with a relatively high prevalence, and syndromes in which the deletion was the major mechanism underlying the pathology. Deletions of these regions are generally not detectable or may be missed by conventional karyotyping. The syndromes have well-described phenotypes and known clinical significance.[11]

Results

We attempted analysis using Prenatal BoBs™ in 70 of the 77 samples collected. Of the remaining seven, six were empty gestational sacs, haemorrhagic samples with MCC or macerated samples with absence of chorionic villi; the last sample was excluded owing to a low DNA amount after DNA extraction (<5 ng/μL).

Cytogenetic analysis using Prenatal BoBs™ gave a conclusive result for 62 of the 70 samples (88.6%). Failure to deliver a result in the remaining eight cases (11.4%) was due to poor DNA quality. Of the 62 samples, 60 were considered normal (96.8%) and two had abnormal results (3.2%) (Table 2): one trisomy 21 (intrauterine fetal death at 11 weeks' gestation), and a deletion of 17p13.3 (Miller-Dieker syndrome, MDS) (Fig. 2).

Standard karyotyping was not attempted in 26 samples, 19 of which were too macerated for cell culture. Karyotyping failed in 38 samples because of absence of cell proliferation and microbial contamination in the cell cultures. Thirteen samples had normal karyotypes.

Trisomy 21 was detected in a female fetus who died *in utero* at 11 weeks' gestation. At autopsy, the fetus was found to have a hygroma and generalised subcutaneous oedema. As the cell culture failed, we could not determine whether the trisomy was total or partial.

The deletion of 17p13.3 (MDS) was identified in a female fetus who died *in utero* at 38 weeks' gestation. MDS is characterised by a developmental defect of the brain (type 1 lissencephaly), which is caused by incomplete neuronal migration. This microdeletion syndrome is also characterised by distinctive facial features and other congenital malformations. Serum markers in the first and second trimester were normal and the second ultrasound scan revealed a single umbilical artery. At autopsy, the fetus was found to be hypotrophied and short in length (<25th percentile) and

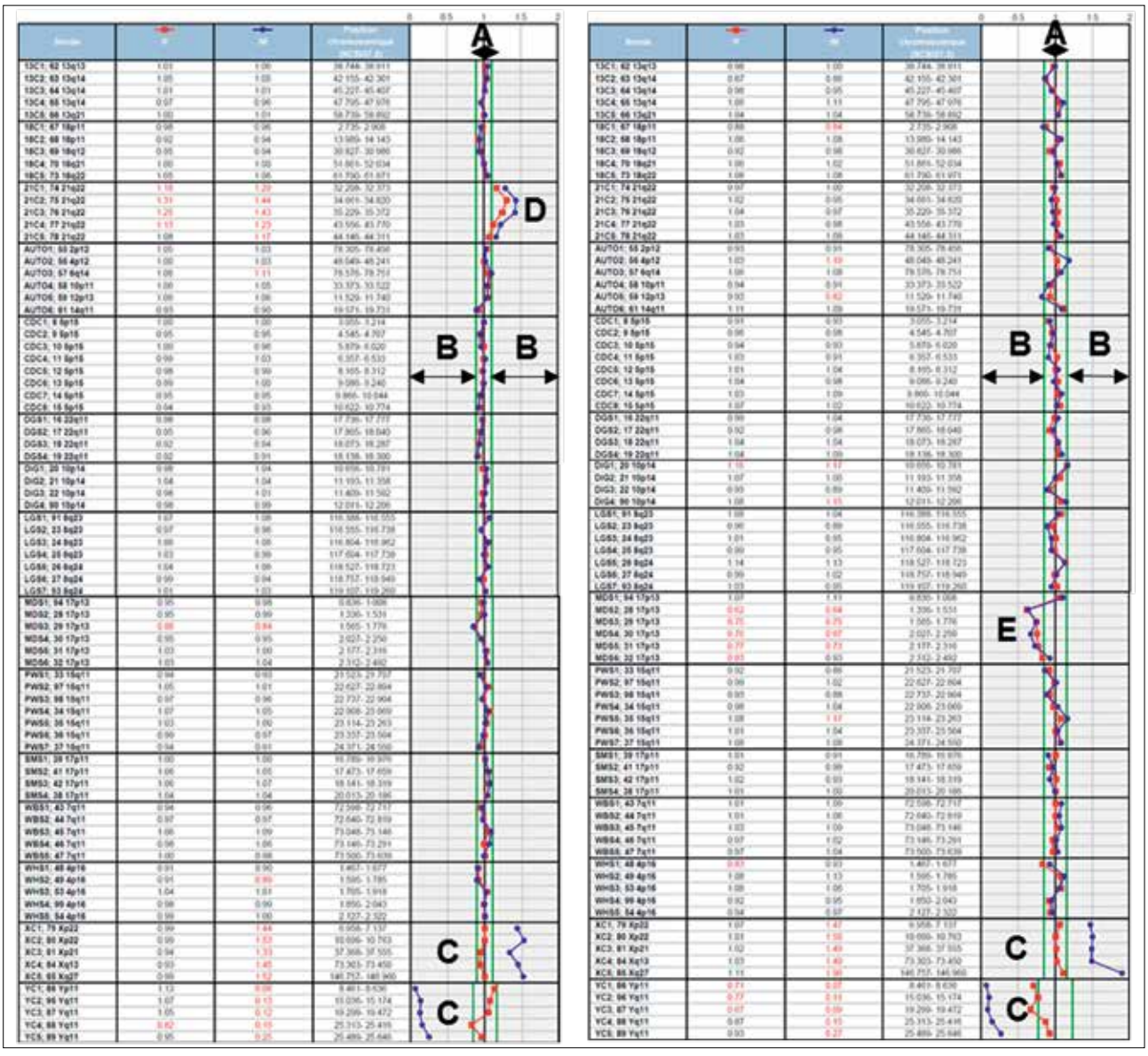

Fig. 2. Prenatal BoBs™ profiles of case 1 (left) and 2 (right). Red spots show sample-to-female references ratios and blue spots sample-to-male references ratios. Arrows show the range of (A) normal ratios, and (B) abnormal ratios. C = female gonosome profile showing that the patient has the same number of copies of chromosome X as female references (red dots in the normal range) but more than male references (blue dots in amplification), and has the same number of copies of chromosome Y as female references (red dots in the normal range) and less than male references (blue dots in deletion). D = 21q22 amplification showing trisomy 21 profile. E = 17p13.3 deletion.

Table 2. Abnormal results of the Prenatal BoBs™

Cases	Maternal age (years)	Gestational age (weeks)	Type of sample/ fetal outcome	BoBs result	Karyotype result
1	42	11	Biopsy/IUFD	47,XX,+21	Failure
2	25	40	Placenta/IUFD	46,XX,del 17p13.3	46,XX

IUFD = intrauterine fetal death.

to have a decreased head circumference (<3rd percentile). However, there were no facial dysmorphic features or cardiac, pulmonary or renal abnormalities. Neuroanatomical examination did not show lissencephaly. FISH analysis showed that the *LIS1* gene, monosomy of which is responsible for MDS, was not deleted. The deletion was characterised further by array genomic comparative hybridisation (4 × 180K, Agilent, USA), which showed a deletion of 2.3 Mb encompassing 40 genes. This deletion, which is not typical of the MDS, was probably responsible for the short stature of the fetus, but the fact that the single umbilical artery could have contributed to death could not be excluded.

Discussion

Prenatal BoBs™ is a recently developed molecular cytogenetic screening tool for the most common aneuploidies (chromosomes 13, 18, 21, X and Y) and the nine most frequent microdeletion syndromes found in POC, which are not detectable or may be missed by conventional karyotyping. This rapid targeted assay is dependent on DNA extraction and does not require live or intact cells, and represents an interesting alternative to conventional cytogenetics that require cell culture.[5,9,11,12] In our study, cell culture failure prevented standard karyotype analysis of 38 samples. However, Prenatal BoBs™ provided a conclusive result in all cases, making it a useful tool for the cytogenetic analysis of POCs, especially when culture fails.

We analysed 70 DNA samples extracted from chorionic villi of products of SM, skin fibroblasts, amniotic cells and products of curretage. Eight cases were uninterpretable, giving a technical failure rate of 11.4%, which was higher than the rate of ~3% registered for prenatal screening using Prenatal BoBs™[5,12] and also higher than the rate of ~ 2% reported for the screening of POCs using Karyolite Bobs™.[6,10] These results may be explained by the fact that we had numerous samples with poor DNA quality due to advanced maceration of fetal tissue.

Among the remaining cases, Prenatal BoBs™ revealed the presence of two chromosomal abormalities: one case of trisomy 21 that could not be detected by karyotyping because of cell culture failure and was associated with advanced maternal age (42 years), a risk factor well known to increase the risk of aneuploidies, mostly trisomies,[7] and one case of microdeletion of 17p13.3 (2.3 Mb) that could not be detected by karyotyping because of its low resolution, or by Karyolite BoBs™ because of limitations in the detection of structural rearrangements. These structural rearrangements account for ~6% of abnormalities found in POC.[6] MDS is a rare malformation syndrome manifested by type I lissencephaly and characteristic facial features and associated with a microdeletion of chromosome 17pl3.3, which can be detected by high-resolution cytogenetic techniques in ~50% of cases.[13] Cytogenetic investigation of 1 599 prenatal samples using Prenatal BoBs™ revealed 11 cases of microdeletions and microduplications (0.75%), among which deletion of 22q11.2 (Di George syndrome) was the most frequent abnormality detected.[5]

Prenatal BoBs™ is a targeted assay, so the loci of the genome that may have clinical relevance in an unbalanced state and that are not targeted by the probe set will go undetected.[5,9,11] Better coverage throughout the genome would lead to the detection of additional clinically relevant imbalances, but would also identify gains or losses of unknown or unclear clinical significance.[11,14] Moreover, Prenatal BoBs™ has some limitations in the detection of polyploidies (triploidies and tetraploidies),[5,9] which account for nearly 16% of abnormalities found by conventional karyotyping of POC.[6]

Another disadvantage of SM products is the high rate of MCC. Only a few studies have tested the ability of Prenatal BoBs™ to identify mosaicism and MCC. It has been shown that Prenatal BoBs™ can detect mosaicism in fetal tissue at a level of 20 - 40% abnormal cells or higher,[9,15] and that MCC in the fetal tissue becomes apparent at a level of 20 - 30% of normal female cells.[11]

Prenatal BoBs has been described as more informative than rapid FISH and QF-PCR, which screen only frequent aneuploidies.[9] This technique also enabled us to provide rapid results with conclusive outcomes within 24 hours of receipt of the sample,[5,9,11,12] which is about half the time of fast FISH aneuploidy screening, because it is possible to interpret the profiles of several samples in few minutes.[5,9] Furthermore, it is cheaper than other technologies that are able to detect more alterations (array-CGH), being of the same order of cost as fast FISH.[9,12]

Conclusion

On analysis of 77 samples of POC collected, we found the cause of fetal death in two cases using Prenatal BoBs™ technology. One of these fetuses had trisomy 21 and the other a 17p13.3 deletion, which were missed by karyotyping (owing to culture failure in one case and low resolution in the second). Prenatal BoBs™ appears to be fast, sensitive and a good alternative to other conventional technologies such as karyotyping in the routine screening of SM products, especially when tissue is damaged and macerated, preventing cell culture. In combination with other molecular approaches, use of Prenatal BoBs™ could improve detection of chromosomal abnormalities in POCs and prove helpful in parental counselling.

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